Exhibit 3

Early Prophylaxis With Recombinant Human Interleukin-11 Prevents Spontaneous Diabetes in NOD Mice

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We evaluated the effects of recombinant human (rh) interleukin (IL)-11 on the development of spontaneous and cyclophosphamide-induced diabetes in female NOD mice. Prolonged treatment with rhIL-11 10 μg i.p. five consecutive times a week between the 4th and 22nd weeks of age significantly suppressed both development and cumulative incidence of type 1 diabetes. Disease protection was transient because most of the animals developed type 1 diabetes within 3 months of treatment withdrawal. In contrast, rhIL-11 failed to prevent type 1 diabetes when administered for the first time to euglycemic 18-week-old NOD mice. Most likely, this discrepancy was not due to age-dependent differences in the immunological responses of NOD mice to rhIL-11 because staphylococcus aureus enterotoxin B–induced tumor necrosis factor (TNF) and IL-12 production were equally suppressed by rhIL-11 in 12- and 25-week-old NOD mice. Relative to controls, NOD mice pretreated with rhIL-11 also showed significantly diminished blood levels of TNF, interferon-γ, and IL-12 induced by anti-CD3 antibody and/or lipopolysaccharide. The results demonstrate that rhIL-11 has powerful anti-inflammatory effects that are capable of down-regulating early immunodiabetogenic pathways in NOD mice. *Diabetes* 48:2333–2339, 1999

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Ab, antibodies; ANOVA, analysis of variance; CY, cyclophosphamide; ELISA, enzyme-linked immunosorbent assay; GVHD, graft versus host disease; IFN, interferon; IL, interleukin; IS, insulitis score; LPS, lipopolysaccharide; mAb, monoclonal antibodies; PBS, phosphate-buffered saline; rh, recombinant human; SEB, staphylococcus aureus enterotoxin B; TNF, tumor necrosis factor.

Type 1 diabetes is defined by hyperglycemia that is induced by an inflammatory autoimmune attack that destroys the pancreatic β-cells (1). Type 1 proinflammatory cytokines, such as interleukin (IL)-1, IL-2, IL-12, interferon (IFN)-γ, and tumor necrosis factor (TNF), produced by T- and B-cells and macrophages infiltrating the islets (insulitis) may play a major pathogenetic role either by directly influencing β-cell function, viability, and immunogenicity and/or by recruiting and activating further anti–β-cell immune effectors (2,3).

The deleterious action of type 1 cytokines on type 1 diabetes pathogenesis seems to be counteracted by endogenous anti-inflammatory mediators. These include type 2 cytokines, such as IL-4, IL-10, and IL-13, and type 3 cytokines, such as transforming growth factor (TGF)-β, which block the production and action of type 1 cytokines and naturally occurring cytokine inhibitors, such as IL-1 receptor antagonist, soluble TNF receptors, and anti-cytokine antibodies (Ab) (4). Hence, the development of type 1 diabetes may depend on a fine, perhaps genetically determined, balance between pro- and anti-inflammatory cytokines. Indeed, type 1 cytokine inhibitors (e.g., neutralizing monoclonal Ab [mAb] and soluble cytokine receptors) and type 2 cytokines themselves have antidiabetogenic actions in type 1 diabetic rodent models (BB rats, NOD mice) (1–4).

IL-11 is a member of the IL-6 cytokine family that possesses pleiotropic effects both inside and outside of the immune system (5,6). The cytokine is produced by several cells within the central nervous system, the thymus, the lungs, bones, skin, and connective tissue. Like IL-6, leukemia inhibitory factor, and cardiotrophin-1, IL-11 uses gp130 as the signaling receptor unit (5,6).

Although IL-11 was originally described for its effect on the hemopoietic system (7), recent studies have demonstrated that IL-11 acts also on T- and B-cells and macrophages (8–10). The powerful anti-inflammatory effect of IL-11 is at least partially mediated by its capacity to inhibit the production of the proinflammatory cytokines TNF, IL-1β, IL-12, and IFN-γ; this effect is probably secondary to inhibition of nuclear translocation of NF-κB, which is caused by IL-11–induced increases in the levels of the NF-κB inhibitors I-KBα and I-KBβ (11).

The monocyte/macrophage has been proposed as the primary target through which IL-11 exerts its immunomodulatory effect. Central to the capacity of IL-11 to inhibit the pro-

duction of IFN-γ from T-cells is its suppressive effects on the production of IL-12 by macrophages, which subsequently induces a Th1-polarized response (4,8). However, more recent evidence indicates that IL-11 also promotes Th2 polarization with augmented production of the type 2 cytokine IL-4 (10). These findings suggest IL-11 is of potential importance in the treatment of cell-mediated type 1 cytokine-dependent immunoinflammatory diseases; this possibility prompted us to study the effects of exogenous IL-11 on the clinical, histological, and immunological parameters during the development of type 1 diabetes in the NOD mouse.

RESEARCH DESIGN AND METHODS

Animals and reagents. NOD mice of both sexes were purchased from Charles River (Calco, Italy). They were kept in laminar flow hoods and bedding with free access to autoclaved food and water. At the beginning and end of the study, blood samples from at least five sentinel mice per group always tested negative for major murine pathogens, including Sendai virus, mouse hepatitis virus, and mycoplasma pulmonis (Immunocomb kit, Charles River). When kept under these experimental conditions, there were no significant differences in the mean ages of onset or the cumulative incidences of diabetes (from ~70 to 85% at 35 weeks of age) in more than 500 control (PBS-treated or untreated) female NOD mice obtained in the last 4 years from Charles River.

Recombinant human (rh) IL-11 purified from *Escherichia coli* was manufactured at the Genetics Institute (Cambridge, MA) and had a specific activity of 1.5×10^5 U/mg as determined by T10 proliferation assay. rhIL-11 has been previously shown to be equally active in mice and rats (8–10,12–22). rhIL-11 was dissolved in sterile PBS and injected intraperitoneally into mice in a final volume of 100 µl. Three groups of NOD mice served as controls for the repeated injections with rhIL-11. One group was treated with PBS, another with *E. coli*–derived rhIFN-α (Hoffman-La Roche, Basel), and the last with rhIL-11 that was boiled for 30 min, which is known to abrogate the biological activities of the cytokine. Concanavalin A (ConA) and lipopolysaccharide (LPS) serotype 055:B5 were purchased from Sigma Chimica (Milan, Italy). Staphylococcus aureus enterotoxin B (SEB) was purchased from Toxin Technology (Sarasota, FL). Cyclophosphamide (CY) was purchased from Schering Plough (Milan, Italy). Hamster anti-mouse CD3 mAb and solid-phase enzyme-linked immunosorbent assay (ELISA) kits for detection of mouse IL-4, IFN-γ, and TNF were purchased from Pharmingen (San Diego, CA). ELISA kits for mouse IL-12 were purchased from Endogen (Cambridge, MA). Samples were run in duplicate according to the manufacturers' instructions. The lower limits of sensitivities of the assays were 7 pg/ml for IL-4 and IFN-γ, 10 pg/ml for TNF, and 5 pg/ml for IL-12. Intra- and interassay coefficients of variations were between 12 and 18%. To calculate mean values, samples with cytokine values below the levels of detection were assigned the limits of sensitivity of the assay as a theoretical value.

Experimental design

Spontaneous type 1 diabetes. Euglycemic female NOD mice were randomly allocated into different groups receiving the treatments described below, starting at the 4th or 18th week of age. Because insulitis is virtually absent in 4- to 6-week-old NOD mice and is actively ongoing in most animals at 18 weeks of age(23), this approach allowed us to investigate the effects of exogenous rhIL-11 in both the early and late diabetogenic stages.

For the early prophylactic treatment, 4-week-old mice received 10 µg rhIL-11, an equal volume of PBS, 50,000 U *E. coli*–derived rhIFN-α, or 10 µg heat-inactivated rhIL-11. Treatments were given intraperitoneally five consecutive times a week until the age of 22 weeks. The mice were screened for diabetes development twice a week by means of glycosuria. If the mice tested positive, measurements of glycemia were also taken. Mice were diagnosed as diabetic when fasting glycemia was >11.8 mmol/l for 2 consecutive days. At the end of the study period, all remaining euglycemic mice were killed, and the pancreases were examined for insulitis severity.

In a second set of experiments, 4-week-old mice were similarly treated with either rhIL-11 or PBS alone until the age of 22 weeks. At this time point, treatment was interrupted, and the mice were monitored for diabetes development until the age of 50 weeks.

For the late prophylactic treatment, 18-week-old mice were randomly divided into two experimental groups: one group was treated with 10 µg rhIL-11 ($n = 20$); the other group was treated with PBS alone ($n = 20$). Treatment was given five consecutive times a week for 17 consecutive weeks until the age of 35 weeks. Mice were then screened for type 1 diabetes development as described above. Pancreatic specimens were collected from diabetic mice that were killed at diabetes onset or from the remaining euglycemic mice at the end of treatment.

CY-induced type 1 diabetes. Another set of experiments was performed to evaluate the effects of rhIL-11 on the accelerated model of diabetes that can be provoked in 14- to 18-week-old NOD mice by challenge with one or two large doses of CY at 2-week intervals. For this purpose, 18-week-old female NOD mice were injected intraperitoneally with 250 mg/kg body wt of CY and randomly divided into two experimental groups: one treated with rhIL-11, and the other treated with PBS alone. Starting 1 day before CY challenge, the mice were treated every day with either rhIL-11 or PBS, five times a week, until day 14. The mice were screened for diabetes development on days 7, 14, and 16 and diagnosed as diabetic when the fasting glucose levels were >11.8 mmol/l for 2 consecutive days.

Spleen cell transfer of type 1 diabetes. Six-week-old euglycemic male NOD mice were irradiated with 650 Rad before intravenous injection of 2×10^7 spleen cells from acutely diabetic female NOD mice (24). Another group of NOD mice received additionally the same amount of spleen cells from euglycemic NOD mice treated with rhIL-11 from the 4th to 22nd weeks of age, as described above.

Ex vivo effects of rhIL-11 on SEB-induced IL-12 and TNF production. A 10-µg dose of rhIL-11 or PBS were administered intraperitoneally to 12- or 25-week-old female NOD mice (8 animals per group), respectively, at times −24, −1, and 18 h relative to the injection of 100 µg SEB. Peritoneal cells were recovered 24 h after SEB injection and cultured without stimulation for 24 h as described (25). IL-12 and TNF in culture supernatants were quantified by the same solid-phase ELISAs as previously described.

In vivo effects of rhIL-11 on anti-CD3– and LPS-induced increase in the blood levels of IL-4, IL-12, IFN-γ, and TNF. Female NOD mice between the ages of 8 and 11 weeks were treated with either 10 µg of rhIL-11 or PBS before the intraperitoneal injection of 10 µg of anti-mouse CD3 mAb or 500 µg LPS. Blood samples were collected from 10 mice in each group that were killed without being injected with anti-CD3 mAb or LPS, or 2 and 6 h after being injected with either of them. After the blood clotted at room temperature, the serum was immediately separated by centrifugation at 1,000*g* and stored at −20°C until cytokine measurements were taken.

Histological examination of pancreatic islets. Samples were fixed in Bouin's solution and embedded in paraffin for light microscopy. Serial sections (5 µm thick) were stained with hematoxylin-eosin for general morphology. For the semiquantitative evaluation of infiltration, only sections containing eight or more islets were selected, and at least eight islets per pancreas were evaluated in a blind fashion by an observer unaware of the treatment or the status of the mice as detailed (26). The degree of mononuclear cell infiltration was graded as follows: 0, no infiltrate; 1, periductular infiltrate; 2, peri-islet infiltrate; 3, intraislet infiltrate; and 4, intraislet infiltrate associated with β-cell destruction. The mean score for each pancreas was calculated by dividing the total score by the number of islets examined.

RESULTS

Lack of toxicity of rhIL-11 in NOD mice. Treatments with rhIL-11, heat-inactivated rhIL-11, and rhIFN-α were well tolerated. Even when therapy was continued to the 22nd week of age, there were no differences among these groups or the PBS-treated control group in body weights, behavior, and general appearance of the animals (data not shown).

Early treatment with rhIL-11 prevents insulitis development and temporarily reduces the cumulative incidence of type 1 diabetes in NOD mice. While acute diabetes with glycosuria and hyperglycemia occurred in almost half of the control NOD mice treated with PBS, *E. coli*–derived rhIFN-α, or heat-inactivated rhIL-11 by the age of 22 weeks, the cumulative incidence of diabetes was significantly reduced by rhIL-11 (Fig. 1). Histological analyses of pancreatic β-cells from the remaining euglycemic mice from either rhIL-11 ($n = 22$) or PBS ($n = 14$) treatment groups at 22 weeks of age also revealed that rhIL-11 ameliorated the insulitis process. Thus, most (11 of 14) of the PBS-treated control mice had actively ongoing insulitis, which varied from peri-islet infiltrate (grade 2) to intraislet infiltrate associated with β-cell destruction (grade 4) (insulitis score [IS] = 2.4 ± 0.8, mean ± SD). In contrast, all the rhIL-11–treated mice exhibited an insulitis process characterized by periductular or peri-islet infiltrate (IS 1.2 ± 0.72; $P < 0.0001$, analysis of variance [ANOVA] with Bonferroni's adjustment). The insulitis

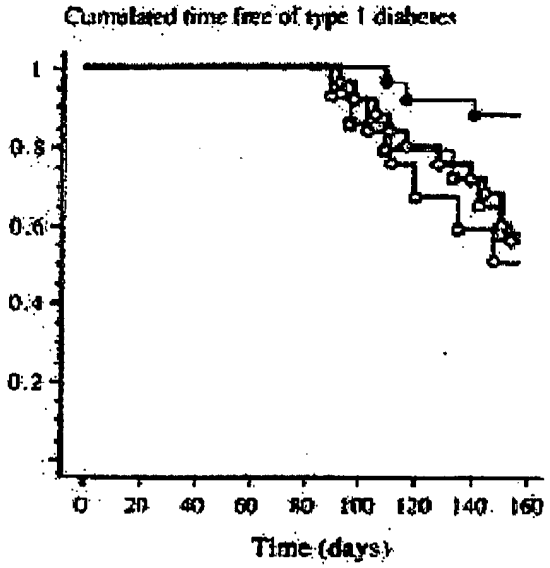

FIG. 1. Prevention of type 1 diabetes in NOD mouse by early prophylaxis with rhIL-11. Euglycemic female NOD mice at 4 weeks of age were treated intraperitoneally with 10 μg rhIL-11 (●, n = 25), 10 μg heat-inactivated rhIL-11 (○, n = 12), 50,000 U rhIFN-α (□, n = 14), or 100 μl PBS (◇, n = 25) five consecutive times a week until the age of 22 weeks. The results depicted with rhIL-11 and PBS are merged from three independent experiments, and those from rhIFN-α are merged from two independent experiments. The experiment with heat-inactivated rhIL-11 was performed only once. Variability among independent experiments was in each case <10%. rhIL-11 vs. PBS: P = 0.014.; rhIL-11 vs. heat-inactivated rhIL-11:, P = 0.009. rhIL-11 vs. rhIFN-α: P = 0.027. The other possible comparisons all have P > 0.05 by Logrank (Mantel-Cox).

process of 22-week-old euglycemic NOD mice treated with either heat-inactivated rhIL-11 (n = 6, IS 2.3 ± 0.9) or rhIFN-α (n = 8, IS 2.6 ± 1) was very similar to that observed in PBS-treated control mice.

A second set of experiments was conducted to ascertain whether the antidiabetogenic effect of rhIL-11 was long-lasting. Two groups of euglycemic female NOD mice were created and treated with either rhIL-11 or PBS exactly as described in the first experiment. The mice that remained euglycemic at the interruption of the treatment at age 22 weeks were followed for type 1 diabetes development until 50 weeks of age. As shown in Fig. 2, the protection was lost 2–3 months after treatment withdrawal when the cumulative incidence of the disease was similar to that of control mice.

Late treatment with rhIL-11 does not influence insulitis or type 1 diabetes development. In contrast to what was seen with early prophylaxis, late prophylaxis with rhIL-11 failed to influence the natural course of type 1 diabetes in NOD mice. In fact, during the treatment period, 15 of 20 mice treated with rhIL-11 developed diabetes as compared with 16 of 20 mice treated with PBS. Age at type 1 diabetes onset was also similar between the two groups (mean age ± SD 180 ± 33 vs. 171 ± 30, respectively). The extent of insulitis was also similar in controls and rhIL-11–treated mice (IS 3.3 ± 0.5 and 3.2 ± 0.8, respectively).

rhIL-11 fails to prevent CY-induced type 1 diabetes. Although the precise mechanism by which CY accelerates and synchronizes the development of autoimmune diabetes is unknown, inhibition of suppressor cell functions is thought to play a role that allows autoreactive cells to destroy β-cells within 2–4 weeks; this effect is IFN-γ– and IL-1β–dependent (26,28–31). Accordingly, type 1 diabetes developed in the majority of NOD mice within 15 days of CY challenge (Table 1). There were no significant differences in the kinet-

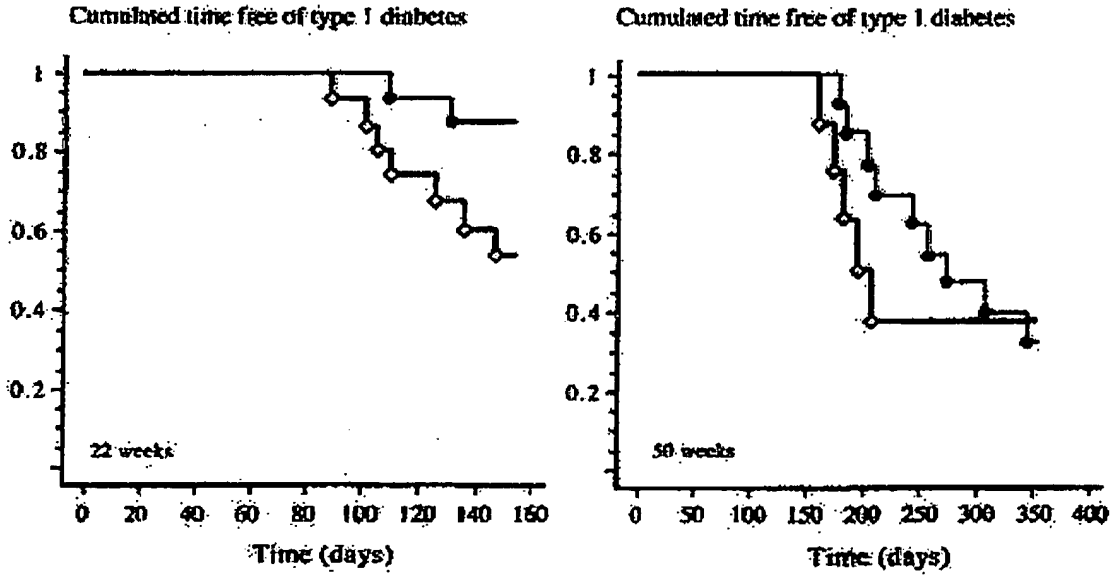

FIG. 2. Loss of IL-11–induced protection after treatment withdrawal. Mice were treated as shown in Fig. 1 with either rhIL-11 (●) or PBS (◇). Each group consisted of 15 mice. After 22 weeks, the treatment was withdrawn, and the mice were followed for diabetes development until 50 weeks of age. At 22 weeks of age, P = 0.048 vs. controls by Logrank (Mantel-Cox). The results are merged from two independent experiments. Variability between independent experiments was in each case <10%.

TABLE 1
Lack of effect of IL-11 on CY-induced diabetes

Treatment	Diabetes incidence
PBS	9/15 (60%)
IL-11	11/15 (73%)

Euglycemic 18-week-old NOD mice were treated until 2 weeks after CY injection as described in RESEARCH DESIGN AND METHODS.

ics of type 1 diabetes development or in the cumulative incidence between controls and rhIL-11–treated mice.

Early prophylaxis with rhIL-11 does not depend on generation of suppressor cells. Four weeks after injection of spleen cells from acutely diabetic NOD mice, type 1 diabetes occurred in 10 of 10 (100%) recipient male NOD mice. The capacity to transfer type 1 diabetes was not influenced by cotransferring the recipient mice with spleen cells from mice protected by early rhIL-11 prophylaxis: 8 of 10 (80%) developed type 1 diabetes with a similar kinetic to that of control mice. The slight reduction in diabetes incidence did not depend on the pretreatment because 80% incidence of diabetes was also observed in NOD mice preinjected with spleen cells from PBS-treated euglycemic female NOD mice.

rhIL-11 suppresses ex vivo release of IL-12 and TNF from peritoneal mononuclear cells. To test the possibility of different age-dependent responses of NOD mice to the immunomodulatory effects of IL-11, an experiment was conducted where 12- and 25-week-old euglycemic female NOD mice received rhIL-11 or PBS as controls. Peritoneal macrophages were recovered and cultured for secretion of IL-12 and TNF. Cells from mice not treated with SEB did not secrete measurable amounts of the cytokines (Table 2). In contrast, both cytokines were secreted from cells of the SEB-treated mice, and this secretion diminished in cultures from animals pretreated with rhIL-11 (Table 2). The suppressive effect of rhIL-11 on IL-12 and TNF secretion was the same in 12- and 25-week-old mice.

rhIL-11 reduces the blood levels of IL-12, IFN-γ, and TNF induced by anti-CD3 mAb and LPS without modifying anti-CD3–induced release of IL-4. The ability of rhIL-11 to interfere with the in vivo induction of cytokines was tested in mice injected with hamster anti-CD3 mAb or LPS. While anti-CD3 mAb induces release of T-cell–derived cytokines (e.g., IFN-γ and IL-4), LPS primarily provokes the release of macrophage-derived cytokines (e.g., IL-12 and TNF) and, subsequently, T-cell– and NK cell–derived cytokines (e.g., IFN-γ). Before the anti-CD3 mAb and LPS injections, the blood levels of IL-4, TNF, and IFN-γ were all below the limit of sensitivity of the assays in NOD mice, regardless of whether they were treated with rhIL-11 or PBS (Figs. 3 and 4). However, IL-12 could be detected at low levels in the circulation of 3 of 10 PBS-treated mice. The cytokines were massively released into the circulation 2 and/or 6 h after anti-CD3 mAb or LPS injections (Figs. 3 and 4). Mice receiving rhIL-11 showed reduced anti-CD3–induced blood levels of IFN-γ 2 h (1,096 ± 841 vs. 6,314 ± 2,415 pg/ml) and 6 h (1,890 ± 669 vs. 6,290 ± 1,567 pg/ml) after challenge (Fig. 3). The TNF levels were reduced at 2 h (17 ± 13 vs. 55 ± 16 pg/ml) (Fig. 3), as were the LPS-induced blood levels of IFN-γ and IL-12 (Fig. 4). While the levels of IFN-γ were significantly reduced both at 2 h (<7 vs. 76 ± 65 pg/ml) and 6 h (1,431 ± 907 vs. 6,541 ± 1,098 pg/ml), those of IL-12 were significantly reduced 2 h (24.1 ± 10.6 vs. 64 ± 40.7 pg/ml) but not 6 h after LPS injection (Fig. 4). There was no difference in anti-CD3–induced blood levels of IL-4 (Fig. 3). The latter data are consistent with ex vivo studies showing that prolonged treatment with rhIL-11 from the 4th to the 22nd weeks of age failed to modify the spontaneous or ConA-induced release of IL-4 (data not shown).

DISCUSSION

The present data demonstrate that prolonged administration of rhIL-11 to NOD mice prevents the development of insulitis and diabetes, provided that the treatment is instituted in the early stages of the prediabetic period between 4 and 5 weeks of age. In fact, rhIL-11 did not influence spontaneous or CY-induced diabetes when its application was initiated in 18-week-old NOD mice. This beneficial effect was probably not the result of the repeated injections of an immunogenic (human into mouse) *E. coli*–derived protein because the same early prophylactic treatment of NOD mice with either heat-inactivated rhIL-11 or rhIFN-α failed to influence both the incidence and kinetics of type 1 diabetes in these animals. Although *E. coli*–derived rhIFN-α is not very effective in mice, the human cytokine is immunogenic because high titers of anti-human Ab were found in sera of mice treated with either rhIL-11 or rhIFN-α at the end of the study (data not shown). That prolonged treatment with human cytokines is per se insufficient to prevent diabetes development in NOD mice is in accord with previous studies in which neither human IFN-α nor human IFN-γ modulated the development of diabetes in these mice (32). The antidiabetogenic effect of human TNF-α (33) in NOD mouse has been confirmed with mouse TNF-α (34).

TABLE 2
In vivo effect of IL-11 on SEB-induced IL-12 and TNF production

Treatment	12-Week-old mice		25-Week-old mice	
	IL-12 (pg/ml)	TNF (pg/ml)	IL-12 (pg/ml)	TNF (pg/ml)
PBS	31 ± 15	531 ± 218	41 ± 27	421 ± 312
IL-11	12 ± 13	65 ± 44	15 ± 12	76 ± 52
P	0.018	<0.0001	0.026	0.008

Data are means ± SD. NOD mice (eight per group) were treated at times −24, −1, and 18 h relative to the injection of 100 µg SEB. Peritoneal mononuclear cells were recovered 24 h after SEB injection and cultured in vitro without further stimulation for 24 h. In NOD mice not receiving SEB, the IL-12 and TNF levels were below the assay detection limits (5 and 10 pg/ml, respectively). *P* values were determined by ANOVA with Bonferroni's adjustment.

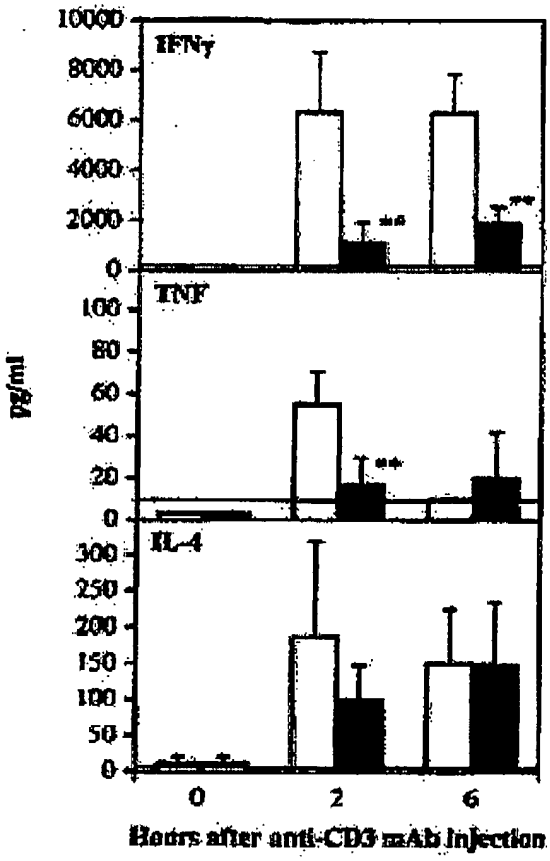

FIG. 3. In vivo effects of IL-11 on anti-CD3 mAb–induced IFN-γ, TNF, and IL-4 production. Euglycemic 8- to 11-week-old mice were treated with either rhIL-11 10 μg/mouse (■) or with PBS (□) 1 h before intraperitoneal injection of 10 μg anti-mouse CD3 mAb. Blood samples were collected from 10 mice in each group killed at 0 h without being injected with anti-CD3 mAb or 2 and 6 h after challenging with anti-CD3 mAb. Serum samples were quantified by cytokine ELISAs, and the results are expressed as mean picogram per milliliter ± SD. Horizontal lines show the sensitivity limits of the ELISAs. $^{**}P < 0.0001$ vs. control groups by ANOVA with Bonferroni's adjustment. The data are merged from two independent experiments. Variability between each independent experiment was <10%.

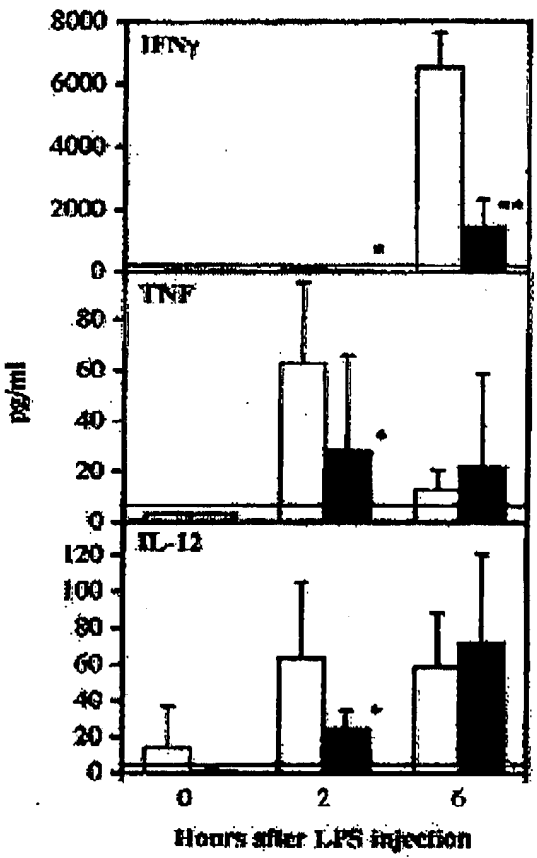

FIG. 4. In vivo effects of IL-11 on LPS-induced IFN-γ, TNF, and IL-12 production. The experiments were carried out as detailed in Fig. 3, except that LPSs (500 mg/mouse) were used to induce cytokines. ■, rhIL-11 treatment; □, PBS-treatment. For IFN-γ, $^{*}P = 0.004$, and $^{**}P < 0.0001$ vs. controls; for TNF, $^{*}P = 0.038$ vs. control; For IL-12, $^{*}P = 0.008$ vs. controls by ANOVA with Bonferroni's adjustment. Horizontal lines show the sensitivity limits of the ELISAs. The data are merged from two independent experiments. Variability between each independent experiment was <20%.

Because insulitis is rarely observed in NOD mice before the age of 4 weeks, and because it rapidly progresses thereafter, affecting virtually all NOD mice by the age of 10–12 weeks (1,23), the results suggest that rhIL-11 exerts its preventive effect only in the early stages of the insulitis process and that interfering with development of insulitis may be central in the antidiabetogenic action of rhIL-11.

The means by which early rhIL-11 treatment prevents insulitis and diabetes in NOD mice might involve generation of suppressor cells or interference with appearance and/or functions of autoreactive effector molecules. The former possibility seems unlikely because cotransfer experiments with spleen cells from rhIL-11–treated NOD mice failed to prevent type 1 diabetes development induced by spleen cells from acutely diabetic animals in syngeneic recipients. In addition, even though the protection afforded by compounds that induce suppressor cells, such as exogenous IL-4, is usually long-lasting and can be maintained up to 6 months after treatment withdrawal (35), most of the rhIL-11–treated NOD mice developed type 1 diabetes within 2–3 months after treatment ended. On the other hand, given the central role of TNF, IFN-γ, and IL-12 in stimulating cell-mediated immune effector functions, the capacity of rhIL-11 to inhibit production of these cytokines may have temporarily impaired the functional activities of autoreactive effectors. In the in vivo study with LPS, however, pretreatment with rhIL-11 only reduced IL-12 blood levels in the mice at 2 but not at 6 h after LPS injection. This finding suggests a weak suppressive action of rhIL-11 on IL-12 synthesis/release in this setting. If so, slight suppression of IL-12 production by rhIL-11 may be a complementary, and not primary, event that contributes to the antidiabetogenic action of rhIL-11 in NOD mice. The capacity of rhIL-11 to suppress LPS-induced secretion of IL-1β and nitric oxide (NO) from LPS-stimulated murine peritoneal macrophages (9) may also contribute to the favorable effect of rhIL-11 in dampening

NOD mouse diabetogenesis as IL-1β and NO are involved in NOD mouse diabetes (30,31,36).

As previously mentioned, IL-11 inhibits nuclear translocation of NF-κB, which is a transcription factor for several genes expressed during inflammatory responses, including TNF, IL-1β, IL-6, and the p40 subunit of IL-12 (11). Stephens et al. (37) demonstrated that inhibition of NF-κB in NIT-1 insulinoma cells protects them from TNF-induced cell death in vitro possibly through reduced expression of the IL-1β–converting enzyme, a member of the caspase pathway of cell death. Thus, besides controlling proinflammatory cytokine production, IL-11 may also control apoptosis that has been proposed to be the mode of β-cell death in type 1 diabetes (38). Thus, inhibition of NF-κB by rhIL-11 could be a central mechanism by which early prophylactic treatment reduces production of proinflammatory cytokines, insulitis, and diabetes in NOD mice.

That rhIL-11 treatment equally suppressed ex vivo secretion of IL-12 in 12- and 25-week-old NOD mice indicates that a different age-dependent response of NOD mice to the immunomodulatory effects of the cytokine was not responsible for the different effects of early and late rhIL-11 treatment. It also suggests that the modifications induced by rhIL-11 on endogenous cytokines (e.g., suppression of TNF, IFN-γ, and IL-12) can only prevent the development of type 1 diabetes during the early stages of the disease. This suggestion concurs with the findings of Trembleau et al. (39), who demonstrated that specific IL-12 inhibitors temporarily prevent NOD mouse type 1 diabetes only when they are administered in the early prediabetic stages. Early blockage of endogenous TNF also prevents the development of type 1 diabetes in these animals without generating suppressor cells (40). Because we have shown that specific IFN-γ inhibitors prevent spontaneous type 1 diabetes when first administered to NOD mice that have advanced insulitis, and because IFN-γ inhibitors are also effective in CY-induced diabetes (26), we believe that the effect of rhIL-11 on IFN-γ production might have been insufficient in this experimental setting.

The capacity of rhIL-11 to suppress TNF, IFN-γ, and, though less effectively, IL-12 secretion upon in vivo and ex vivo conditions agrees with and complements other studies (8–10,13,19,20,22). However, the in vivo modulation of anti-CD3–induced cytokines demonstrates that both macrophages and T-cells are targets for the immunomodulatory effects of IL-11. The inability of rhIL-11 to augment anti-CD3–induced IL-4 blood levels and the ex vivo secretion of the cytokine disagree with recent data by Hill et al. (10), who reported a 10-fold increase in a mouse model of graft versus host disease (GVHD) in IL-4 production from ConA-stimulated splenocytes from rhIL-11–treated animals. The reason for this increase is unknown, but it may be related to the different experimental models. Also, the discrepancy between the NOD mice and the GVHD model may be due to the differences in route and schedule of the drug. For example, Hill et al. administered subcutaneously 250 μg/kg or IL-11 twice each daily. This results in a half-life of the drug of ~6 h vs. ~1 h for intraperitoneal administration.

Although our results show that rhIL-11 is capable of preventing type 1 diabetes in NOD mouse and further indicate the feasibility to halt the development of type 1 diabetes by early antagonism of the type 1 cytokines IFN-γ, IL-12, and TNF, they do not clarify the role, if any, of endogenous IL-11 in the pathogenesis of NOD mouse diabetes. Studies that aim at blockade of endogenous IL-11 with specific inhibitors and that investigate the presence of IL-11 in the insulitic lesions are in progress.

NOD mouse diabetes can thus be included in the increasing list of experimental immunoinflammatory conditions that are favorably modulated by rhIL-11. These include sepsis (13,16,21), arthritides (18,22), and rodent models of inflammatory bowel diseases (15,17,19). Recent data from a phase 1 clinical trial have also demonstrated favorable effects of IL-11 in patients with Crohn's disease (41). Despite these promising observations, and if our data can be transferred to humans, only a minor role can be envisaged for IL-11 in human type 1 diabetes prophylaxis. At present, human prediabetes can be identified only through metabolic and immunologic parameters associated with ongoing β-cell destruction, that is, at a time when rhIL-11 is no longer effective in NOD mouse prediabetes. In addition, the development of type 1 diabetes shortly after rhIL-11 withdrawal suggests that long-lasting therapy is required for any beneficial action of IL-11 on human prediabetes. Nonetheless, it is important to realize that rhIL-11 was used in a mouse model. In mice, rhIL-11 is less effective than mouse IL-11, probably because of low-binding affinity to the mouse IL-11 receptor (42). In addition, prolonged treatment with rhIL-11 induced the production of anti-rhIL-11 Ab that could have reduced the bioactivity of the cytokine. It is therefore possible that mouse IL-11 exerts an even higher antidiabetogenic effect than rhIL-11 in NOD mouse type 1 diabetes and, conversely, that rhIL-11 may modulate immunoinflammatory diabetogenic pathways in humans more powerfully than the data obtained in the present study suggest. The possibility should also be considered that combined prophylactic treatment with IL-11 and other drugs currently in use for human type 1 diabetes prevention, such as nicotinamide and insulin (43), may have synergistic effects. Studies are in progress to test this possibility in the NOD mouse.

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